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A study on the effects of microzooplankton grazing on the diurnal vertical migration (DVM) of Gymnodinium catenatum in the Huon estuary (Tasmania)

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A study on the effects of microzooplankton grazing on the diurnal vertical migration (DVM) of *Gymnodinium catenatum* in the Huon estuary (Tasmania)

Mémoire présenté pour l'obtention du grade de
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Cécile Rousseaux

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Table of contents

List of Figures

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Abstract

The Huon estuary is located in the south-east of Tasmania; over the last two decades there has been a significant increase in aquaculture activity in the Estuary and D'Entrecasteaux Channel, raising concerns about the impact of these activities on the health of the ecosystems and ultimately the ecological sustainability of this industry. Blooms of *Gymnodinium catenatum* occur seasonally (summer and autumn blooms) in the Huon Estuary. The impacts of these blooms are felt in many ways: human health is placed at risk; biogeochemical pathways are altered; and the fishing, aquaculture, and recreation industries suffer substantial economic losses. To reduce the impacts of harmful algal blooms, the understanding of the many factors that regulate the dynamics of HABs (Harmful Algal Blooms) and the manner in which they cause harm is essential.
In this study, we evaluated the impact of the top-down control of zooplankton on phytoplankton by using the dilution technique of Landry and Hassett (1982). Night-time and day-time sampling allowed us to compare the grazing impact throughout a 10 m water column (Port Huon). The characterization of the phytoplankton community was done by combinating a HPLC analysis and a treatment of the results with CHEMTAX. Phytoplankton and microzooplankton was counted for each depth at sampling times T_0 and T+24 h. The results clearly demonstrated that the grazing impact was very high both during night- and day-time and that zooplankton could graze up to 175% of the primary production during the night-time. In order to determine the impact of grazing on different classes of algae, grazing rates were calculated using chlorophyll *a*, fucoxanthin, peridinin and alloxanthin. These results showed that diatoms were most heavily grazed and that similar grazing rates were observed for the peridinin (dinoflagellates) and alloxanthin (cryptophytes) during the night.
A complementary experiment was done to evaluate the influence of grazing on the Diurnal Vertical Migration (DVM) of *Gymnodinium catenatum* by simulating a natural gradient of light in six 1 m-high Perspex cylinders. A large quantity (~60 L) of *G. catenatum* was cultivated and zooplankton, collected from the field, was enclosed in migrating stainless steel cages (pore size = 200 µm). Samples were taken both at noon and at midnight during 216 hours and every two hours during 50 hours at the top and bottom of the cylinders; biomass was evaluated by using a fluorometer. The results from this experiment were rather inconclusive from a statistical point of view.

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A study on the effects of microzooplankton grazing on the diurnal vertical migration (DVM) of *Gymnodinium catenatum* in the Huon estuary (Tasmania)

ROUSSEAUX Cécile

Résumé

L'estuaire Huon est localisé au sud-est de la Tasmanie ; au cours des deux dernières décennies, on a assisté a une augmentation significative de l'aquaculture dans l'estuaire et le canal D'Entrecasteaux, augmentant ainsi l'intérêt de l'impact de ces activités sur la santé de l'écosystème et donc du développement durable de cette industrie. Des blooms saisonniers (bloom d'été et d'automne) de *Gymnodinium catenatum* se développe dans l'estuaire Huon, l'impact de ces blooms est ressenti à différents niveaux : risque pour la santé humaine, altération des écosystèmes et pertes économiques pour les secteurs de la pêche, de l'aquaculture et récréatif. Afin de réduire l'impact de ces HABs (Harmful Algal Blooms), la compréhension des nombreux facteurs régulant cette dynamique, et leur répercussion est essentielle.
Dans cette étude, nous essayons d'évaluer l'impact du contrôle « top-down » du zooplancton sur le phytoplancton en utilisant la technique de dilution de Landry et Hassett (1982). Des échantillonnages fait de jours et de nuit nous permettent de comparer l'impact du broutage dans une colonne d'eau de dix mètres (Port Huon). La caractérisation de la communauté phytoplanctonique a été faite en combinant une analyse HPLC et un traitement des résultats par CHEMTAX. Le phytoplancton et le zooplancton ont été compté pour chaque profondeur à T_0 et à T+24 h. Les résultats démontrèrent clairement que l'impact du broutage, autant durant la journée que durant la nuit, était très important et que le zooplancton pouvait brouter jusqu'à 175.32% de la production primaire pendant la nuit.
Afin d'évaluer l'impact du broutage sur les différentes classes d'algues, les taux de broutage ont été estimés à partir de la chlorophylle *a*, la fucoxanthine, la péridinine et l'alloxanthine. Ces résultats montrent clairement que les diatomées étaient la classe la plus broutée et que des valeurs similaires étaient observées pour la péridinine (dinoflagellés) et l'alloxanthine (Cryptophycés) pendant la nuit.
Une expérience supplémentaire a été conduite afin d'évaluer l'influence du grazing sur la Migration Verticale Journalière (MVJ) de *Gymnodinium catenatum* en simulant un gradient naturel de lumière dans des colonnes en plexiglass de un mètre de haut. Une grande quantité (~60 L) de *G. catenatum* fut cultivée et le zooplancton, récolté sur le terrain, fut placé dans des cages entourées d'un filet (taille de la maille = 200 µm). Des échantillons furent prélevés à midi et à minuit pendant 216 heures et toutes les deux heures pendant 50 heures, à la surface et au fond de la colonne. La biomasse était évaluée en utilisant un fluoromètre. D'un point de vue statistique, les résultats pour cette expérience furent peu concluant.

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Chapter 1: Introduction and objectives

Phytoplankton are floating or weakly mobile microscopic aquatic plants and can be found in both freshwater and seawater. However they can cause important problems when their biomass increases to excess (= bloom). The causes of these blooms can be natural (e.g. related to seasonal increase of nutrient availability) or anthropogenic, resulting from nutrients input by aquaculture, for example. Blooms are not always harmful and can develop without any adverse consequences on the balance of the ecosystem. This study focuses on species, chiefly marine dinoflagellates, producing toxins that can cause illness or death in humans thus having a negative impact on the aquaculture industry.

Harmful algal blooms (HAB) refer to blooms that contain toxins or that cause negative impacts (Smayda 1997). The different symptoms produced by toxic dinoflagellates are Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP) and Ciguatera Fish Poisoning (CFP) (Hallegraeff 1992a).

The toxins (saxitoxins and gonyautoxins) produced by *Gymnodinium catenatum* (see Fig. 1.1) cause PSP. When shellfish, such as oysters, mussels and scallops, ingest *G. catenatum* cells, the toxins are released and the shellfish becomes poisonous for human consumers, birds and other animals (NZFSA 2004). In extreme cases, PSP causes muscular paralysis, respiratory difficulties, and can lead to death.

G. catenatum poses threats to the aquaculture shellfish industries, due to economic losses resulting from farm closure. The ecological, economical and health consequences make the study of these blooms an essential part of managing our aquatic resources.

HABs are a natural phenomenon and have existed since a long time but, human activities on the environment have resulted in an increase in the extent and frequency of algal blooms, making the understanding of HABs even more pressing. Shipping ballast water is now recognized as a pollutant of major potential consequence, with some hundred marine species having been translocated around the world (Rigby and Hallegraeff 1994) and the research is now focused on the management and control measures of this ballast water (e.g. Hallegraeff 1998, Rigby and Hallegraeff 1996, Rigby *et al.* 1993, Bolch and Hallegraeff 1993).

1.1. The blooms: consequences, conditions and solutions

1.1.1.The blooms: introduction

A bloom occurs when algae rapidly increase in numbers to the extent that they become dominant in the local planktonic or benthic community. Such high abundance can result from explosive growth, caused for example, by a metabolic response to a particular stimulus (e.g. nutrients or some environmental conditions like a change in water temperature), or from the physical concentration of a species in a certain area due to local patterns in water circulation.

Although the existence of HAB had already been described in the Old Testament (Exodus 7:21), the incidence of these seemed to have increased over the past decades (Smayda 1997, Hallegraeff 1993). The cause of this trend is probably a greater scientific awareness, and human activity like increase input of nutrient coming from fish farms, changes in the hydrology of rivers, ballast water introduction (Hallegraeff 1992a, Hallegraeff 1992b, National Sea Grant College Program *et al.* 2001). In the sea, blooms are formed by a variety of algae (e.g. the Prymnesiophyte *Phaeocystis* in the North Sea, the diatoms *Cerataulina pelagica* in New Zealand, cyanobacterium *Microcystis aeruginosa* in the Baltic Sea), but dinoflagellates forming red tides are often mentioned in coastal area (e.g. *Gymnodinium* sp., *Alexandrium, Pfiesteria piscicida*).
Gymnodinium catenatum, toxic dinoflagellate, was first recorded from south-east Tasmania in 1985 (Hallegraeff and Sumner 1986). It has produced since then regular blooms in the Huon Estuary and adjacent waters (Hallegraeff *et al.* 1995) causing ecological and economical losses. A bloom of *G. catenatum* is defined as greater than 10,000 cells/L which is the level at which shellfish tend to become toxic (Hallegraeff *et al.* 1995).

1.1.2.The blooms: ecological, economical and health consequences

The toxins (saxitoxins and gonyautoxins) produced, as part of its normal metabolism, by *Gymnodinium catenatum* can have tragic repercussion in different sectors: health, ecology and economy. Marine animals directly affected by these microscopic plants are those that filter their food from the water. Shellfish such as clams, mussels, and oysters use this simple filter-feeding method, and during a bloom, thousands of these tiny plants may be filtered through a shellfish system. Strangely enough, the shellfish grows well on these toxic plants, but concentrates the poison in their tissues at the same time. PSP toxin acts, after ingestion by birds, mammals including humans, within minutes (Chang F.T. *et al.* 1992). The victim may feel a tingling in the lips, a burning sensation in the gums and tongue, and a numbness that spreads from the face to the neck, arms, and legs. In the most severe poisonings, the throat feels constricted and speech become incoherent. Death may follow as a result of respiratory arrest. The toxin is relatively stable and can even survive cooking. Treatment consists in removing as much infected shellfish as possible from the stomach by inducing vomiting. Another treatment, called charcoal hemoperfusion, removes PSP toxins from the blood (Lehane 2000).

The toxicity of the shellfish is measured by a standardized mouse test in which mice are injected with an extract of ground-up shellfish suspected of contamination.

G. catenatum also poses threats to wild and aquaculture shellfish industries, due to economic losses resulting from farm closures. The magnitude of economic loss ranges from short-term to long-term. The shellfish farms have to close when the amount of neurotoxin exceed 80 µg saxitoxin equivalents per 100 g mouse tissue.

1.1.3.The blooms: conditions for development

The HES (Huon Estuary Study) was undertaken in southern Tasmania from 1996 to 1998 and expanded in 2001 based on the need to evaluate the environmental quality and understand the dynamics of the estuary as a system. The HES sampling has demonstrated interannual variability in the magnitude of dinoflagellates blooms. In all years *G. catenatum* was present, reaching peak abundance in summer and autumn but only in some years were blooms observed. The conditions that enable the formation of blooms during these years are still under investigation.

Hallegraeff *et al.* (1995) identified key environmental variables that regulate *G. catenatum* blooms and associated shellfish toxicity in southern Tasmanian waters from 1986 to 1994. In order to determine what stimulates *G. catenatum* bloom formation, they examined historical data of toxicity and hydrological and meteorological data. Their hypothesis is that *G. catenatum* blooms can only develop within environmental constraints, which include a seasonal temperature window from January to June, with major blooms (as shown by high toxicity) only developing when water temperatures are greater than 14°C. A threshold runoff in the weeks preceding it and a calm stable water column during five days or more contribute to the bloom development. They also found no correlation between macronutrients and bloom initiation. However, the HES observed that blooms could develop when water stratification was weak, while strong stratification appeared to enhance bloom intensity and blooms persisted at temperatures below 14°C (CSIRO-Huon Estuary Study Team 2000).

The river runoff is a major influence on the water column stability, and also a source of dissolved organic matter (DOM), which stimulates the growth of dinoflagellates (Doblin *et al.* 1999). The presence of a surface layer of humic rich water during much of the blooms periods may be important for both the dissolved organic matter it contains as well as the water stratification it endows.

In addition, the life history of *G. catenatum*, in particular its resting cyst dynamics may be important in bloom initiation and development. Macronutrients supply does not appear to play a major triggering role, however, the capacity of *G. catenatum* to access both surface and deep nutrients (particularly ammonia) by vertical migration clearly supports blooms.

1.1.4.The blooms: prevention, mitigation and control

Management strategies reduce the impacts of the HAB by preventing their occurrence or reducing their extent; by minimizing HAB impacts on human health, other living resources and coastal economies when they do occur; or by actions which directly reduce, control or contain the bloom population.

The Invasive Species Specialist Group (ISSG) has defined different preventive measures that includes the washing up of all the gears that have been in the water (NZFSA 2004), a more accurate test on the ballast water (e.g. DNA signature, see CSIRO 2004) and the use of different physical treatments (electrical shock, heat treatments, ultra-violet and basic oxygen furnace) on organisms in ballast water.

Examples of mitigation strategies might include moving fish cages from the path of HAB, or reducing the quantity of fish food to minimize their susceptibility to form a bloom.

Approaches to direct bloom intervention (control strategies) fall into three categories: mechanical, physical/chemical, and biological control.
Mechanical control involves the use of filters, pumps, and barriers (e.g. curtains, floating booms) to remove or exclude HAB cells, dead fish, or other bloom-related materials from impacted waters.
Physical and chemical control involves the use of chemical or mineral compounds to kill, inhibit, or remove HAB cells.
Chemicals include copper compounds, barley straw, and chemical oxidant such as chlorine, peroxide, ozone, and chloramines (Chorus and Bartram 1999). The use of clays and other flocculants to remove cells from the water column are potentially more benign than strictly chemical control efforts.
Biological control involves the use of organisms or pathogens (e.g. viruses, bacteria, parasites, zooplankton, shellfish) that can kill, lyse, or remove HAB cells. Viruses, for example, have the potential to be highly specific and effective control agents. However, viruses are sometimes so host-specific that they are unable to infect different genetic strains of the same host species, as often occurs in a HAB (National Sea Grant College Program *et al.* 2001).

Unfortunately each of these strategies presents additional adverse effects on coastal ecosystems that may not be proportional to the benefits gained by their utilization. Therefore, the elimination of specific algae in an area without damaging other species or altering ecosystem functions stays a challenge.

Other strategies include a better monitoring and surveillance to reduce the risk of ingestion or exposure to toxins, an improved forecasting to allow more time to protect resources and avoid risks, the restoration of affected resources and a variety of alternative actions to minimize effects which might occur.
The best approach to minimize the impact of these blooms is still, to find the different factors that enable its formation. As the population is developing, the nutrient input due to human activity is increasing too, often resulting of clearing and farming (increased use of phosphate and nitrogen fertilizers), some intensive farming and sewage. In the case of the Huon Estuary the aquaculture industry itself puts in tons of nitrogen as feed every year.

1.2. Introduction to the dinoflagellates, especially *Gymnodinium catenatum*

Phytoplankton, or microalgae are the main primary producers in aquatic ecosystems. These single-celled organisms produce organic matter by using water, atmospheric carbon dioxide and solar energy (photosynthesis). They reproduce rapidly when the condition of light, temperature, nutrient and salinity are optimal for the species.

These microscopic plants can be differentiated into three size class: the microplankton (20-200µm), the nanoplankton (2-20µm) and the picoplankton (0.2-2µm). In the classification of living organisms, they are protists, autotrophic, mixotrophic or heterotrophic, and belong to several classes of algae; many cyanobacteria are also considered components of the phytoplankton.

The dinoflagellates are a large group of flagellated protists. Most are marine plankton, but they are common in fresh water habitats as well. About half of all dinoflagellates are photosynthetic (autotrophic), and these make up the largest group of algae aside from the diatoms. Heterotrophic species make up the other half, eating other plankton, and sometimes each other, by snaring or stinging their prey.

Most dinoflagellates are unicellular forms with two dissimilar flagella. One of these extends towards the posterior, called the "longitudinal flagellum", while the other forms a lateral circle, called the "transverse flagellum". In many forms these are set into grooves, called the "sulcus" and "cingulum". The transverse flagellum provides most of the force propelling the cell, and often imparts to it a distinctive whirling motion, which is what gives the name dinoflagellate refers to (Greek *dinos*, whirling).
Most dinoflagellates are haploid, and reproduce primarily through fission, but sexual reproduction also occurs. This takes place by fusion of two individuals to form a zygote,
which may remain mobile in typical dinoflagellates fashion or may form a resting cyst, which later undergoes meiosis to produce new haploid cells.
Dinoflagellates sometimes bloom in concentrations of more than a million cells per millilitre. Not all dinoflagellate blooms are dangerous but some species produce neurotoxins, which in such quantities (a million cells per millilitre) kill fish and accumulate in filter feeders such as shellfish, which in turn may pass them on to people who eat them. This phenomenon is called a red tide (or HAB), from the colour the bloom imparts to the water. Some colourless dinoflagellates may also form toxic blooms, such as *Pfiesteria*.

The species here studied, *Gymnodinium catenatum,* is a toxic, bloom-forming species of microalgae. It is usually seen in long, swimming chains of tiny cells, with up to 32 cells in a chain (occasionally 64). It is also seen as solitary cells with a green-brown colour. The size of these cells ranges from 38-53 µm long and 33-45 µm wide. This species, found in bays and estuaries, is widely distributed, from the Mediterranean to the Caribbean, Indian Ocean and Australasian waters. The physical and chemical variables that correspond with the presence of *G. catenatum* are, in descending order of importance: temperature, phosphate, dissolved oxygen, silicate, nitrite, and nitrate (Morquecho and Lechuga-Devéze 2004).

The phytoplanktonic flora is very diverse in south of Tasmania and is often characterized by a high biomass of dinoflagellates (e.g. *Gymnodinium catenatum*, *Ceratium furca, C. tripos, Dinophysis acuminata, Protoperedinium* spp., *Prorocentrum gracile*). *Gymnodinium catenatum* is a dominant species, present in relatively low abundance between July and

December, and occasionally forming blooms from late December to June (CSIRO-Huon Estuary Study Team 2000). The dinoflagellates blooms are interspersed with diatom blooms and they often co-exist (Jameson and Hallegraeff 1994).

1.3. Human impact in the Huon estuary

The first economic activity, which developed in the estuary, was sawmilling. This industry had a harmful effect on the estuary: for the sawmill to operate, an aqueduct had to be constructed in order to wash the sawdust into Hospital bay (Port Huon) and, extensive harvesting of the forest, resulted in a runoff of nutrients and organic matter into the estuary. Moreover this industry was increasing the transport of goods by boat and thus the introduction of species. This industry closed in 1929. A pulp mill developed on the same site (Whale point) in 1962 and discharge continued until July 1991.

Nowadays, the Huon Estuary is the centre for aquaculture in Tasmania; marine farming has grown rapidly since the establishment of shellfish culture in the 1960s and finfish farming in the 1980s. This industry is now taking an important place in Tasmania's economy, almost 80 % (1994) of the State's salmonid production occurs in the Huon estuary and the bay of Port Esperance (Tassal Ltd-*unpublished data*). In 1994, 21 fish farms (Atlantic salmon, mussels and oysters) operated in that area, covering 130 ha (0.6 %) of the total surface area (DPIF 1994) (*in* CSIRO Huon Estuary Study 2000);
Even though the fish industry brings a lot of economical benefits, employment, and is partly compensating the declining catches from the wild fisheries, it is important to know about the impact of these activities on the health of the ecosystem and the ecological sustainability of the industry. The potential impact on the ecosystem is primarily that of eutrophication, and anoxia, which could lead to changes in the biomass and species present in the Huon.

Human practices in the catchment influence the natural processes mostly by increasing the transport of material to waterways, or by adding new inputs to the system. Activities in the waters of an estuary and along its banks also impinge on environmental quality. In rural regions like the Huon, urban inputs are minimal. Point sources are linked to sewage-treatment-plant (STP) outfalls, leachate from tips or waste dumps, or direct discharge from agricultural operations (e.g. dairies and piggeries). The diffuse sources result from wash-off from land recently cleared for forestry or farming. They also result from rainfall running over, and moving through ground that has been treated (with fertilisers, pesticides or other substances) or modified by cropping or other farm activities. In addition, hydrologic modification affecting the natural course of runoff and stream flow, can cause contamination through point source or diffuse inputs.

1.4. Grazing and its impact on phytoplankton community structure

1.4.1. Introduction

The concentration of phytoplankton in a parcel of water is the consequence of a variety of processes which act simultaneously, having a positive or a negative effect on the growth rate of individual plant populations (Downing and Rigler 1984). The definition of the grazing rate, the ingestion rate and the feeding rate are given hereafter and have been taken from Downing and Rigler (1984).

The grazing rate (g) is defined as the volume of food suspension from which a zooplankton would have to remove all cells in a unit of time to provide its measured ingestion. Synonyms of this term are often used in the literature and are, for example, searching rate, filtering rate, filtration rate and clearance rate.

The grazing rate has to be distinguished from the ingestion rate, which is a measure of the mass or energy flow into the animal, expressed in cells ingested individual^{-1}time^{-1}. Finally, the feeding rate, equal to the product of grazing rate and food concentration, is an approximation of the substances, which an animal draws from its environment. Part of the ingested food will be assimilated by zooplankton, contributing to zooplankton production and ultimately to fish production.

1.4.2. Importance of the grazing rate measurement

Some studies have measured grazing rates reaching value of ~100% or more of the phytoplankton daily production (Verity and Smetaček 1996). Grazing obviously affects phytoplankton biomass by removing algal cells that are ingested, but also the phytoplankton community structure, as grazing is often selective (e.g. Thys 2002). This is referred to as "grazer control" or "top-down control".

The discovery of selective grazing and top-down control was a major break through in the understanding of phytoplankton dynamics and bloom formation. In fact where top-down control is of major importance, especially in oceanic environments (Fileman and Burkill 2001), it has provided important new insights on the cycling of matter through the aquatic food chain (Verity and Smetaček 1996).

1.4.3. Method to assess the grazing

The available methods for assessing grazing rates of zooplankton have developed rapidly in the last few decades and the methods available to measure grazing rates by microzooplankton on phytoplankton are here described.
Indirect methods are inferential approaches and use quantifiable characteristics of field samples, such as pigment breakdown products and vacuole contents, as the basis for an index of grazing rate.

Indirect methods include inference from *per capita* rates, in which the measured rates are applied to *in situ* grazer abundances to estimate the grazing impact of an entire taxon or assemblage, and correlation of natural consumer-prey cycles. Direct methods involve manipulation of individual grazer organisms or assemblages. The Landry and Hassett's dilution technique (1982), a direct method to measure assemblage grazing rate, is here detailed as it was the method used in this study.

1.4.3.1.Direct method to measure *per capita* grazing rate

a) *Food removal or counting*

A method to assess the grazing impact is to count cells. The animals are introduced to a suspension of food and the rate of accumulation of food by the animals or its rate of loss from suspension is measured. For this sort of experiment the counting of phytoplankton cells, in presence and absence of zooplankton, needs to be done at the beginning of the experiment (time zero = T_0) and at the end of the experiment (T_{24}) (Thys 2002), a process that makes the technique both time consuming to implement and laborious to evaluate. The incubation time is another restriction, it should be long enough to produce a measurable difference, however sedimentation, cells growing or dying, lack of nutrient resupply and other factors associated with long incubation are know to produce errors in the results (Mourelatos 1989). Ronan and Rubble (1980, *in* Thys 2002) have reviewed the limitations of this technique under the term "bottle effects". This term represents the various artefacts created by the handling, the concentration in the incubation bottles and the possible growth of prey during the incubation.
Bottles effect can be reduced by using larger water volumes (Blomqvist *et al.* 2001), a diffusion chamber (Thouvenot *et al.* 1999) or continuous flows (Voigt and Hülsman 2001, *in* Thys 2002).

Downing and Rigler (1984) have demonstrated that the animals could change their behaviour over the period of the experiment as the concentration of food changed. Furthermore, the zooplankton excretion may stimulate phytoplankton growth during the incubation, making the comparison between the experimental bottles and the controls inappropriate (Gliwicz 1975, Porter 1976, Roman and Rublee 1980, *in* Downing and Rigler 1984).

Even if the results of cell counts can be quite variable, microscope examination allow us to distinguish the species that have been grazed.

b) *Alternative to the microscope counting*

The Coulter counters® and their analogues is another tool used for cell counts. The particles to be counted are suspended in an electrolyte and passed through a small aperture containing an electric field. The advantage of this system is that it gives a more accurate count as well as some measure of size, in a relatively short time. However an important disadvantage is that Coulter counter cannot distinguish two species of similar size, meaning that when they are used for natural population, interpretation is much more complicated (see MacDonald *et al.* 1996). Moreover, they do not work for chain species. The Coulter counter is so a better method than visual counting if the sample consists of a single species that happens to be single-celled.

The HPLC pigments and carotenoid analysis represent another alternative to the traditional counting of algae (Head and Harris 1994, Meyer-Harms and von Bodungen 1997, Descy *et al* 1999, *in* Thys 2002). Coupled with absorbance and/or fluorescence spectroscopy, HPLC can accurately separate and quantify pigments at extremely low detection levels within monotypic and mixed algal samples (Wright *et al.* 1991, Mantoura and Llewellyn 1983). These technologies provide a means for facilitating rapid characterization of pigments diagnostic for phylogenetic groups and for monitoring changes in community composition.

c) *Fluorescence labelled algae*

The use of visually detected prey to study the grazing rates and behaviour of microzooplankton has evolved over the years from dye particles (Seaman 1961), to inert fluorescence particles (Borsheim 1984, McManus and Fuhrman 1986), and finally to the fluorescently labelled, heat-killed algae (FLA) (Sherr *et al.* 1987, Rublee and Gallegos 1989, Pace *et al.* 1990) and live fluorescent-stained cells (Landry *et al.* 1991, Monger and Landry 1992, Putt 1991).

Live cells with distinctive characteristics (morphology, pigments) may be used without staining in this method (e.g. *Alexandrium catenella*).

Bacteria and phytoplankton are labelled with an epifluorecent stain and the cells can be either used living or heat-killed. Method to create fluorescently-labeled bacteria (FLB) and algal (FLA) cells were developed by Sherr and Sherr (1987) and Rublee and Gallegos (1989) respectively. The general procedure when using dead preys is as follow: the stained cells are concentrated by centrifugation, decanted and washed with saline, resuspended in buffer and vortexed. Aliquots are transferred to plastic vials and the concentration of FLA (or FLB) is determined using epifluorescence microscopy. The use of live, stained prey cells is an emerging technique, the labelled prey are here added to the *in situ* assemblage containing the consumers (Harris *et al.* 2000) and incubated in the dark for a period of hours. The control treatment consists of the same volume of cell-free filtrate from stained cultures added to the assemblage. Stained cells in consumer's vacuole are enumerated using an inverted microscope equipped for both transmitted light and epifluorescence.

This technique has obvious advantages for visual confirmation of grazing and for detailed, taxa-specific and behavioral study. However, the limits of this technique are the high variability in the fluorescence properties of phytoplankton cells and the grazer discrimination between fluorescently labelled analogs and natural prey (Pace and Bailiff 1987, Sherr *et al.* 1987).

d) *Radioisotopes tracers*

The use of radioisotope labelling permits a very exact measurement of the trophic transfer in the food chain. The frequently used radioisotopes include ^{14}C, ^{3}H and ^{32}P; the first one is mostly used with ^{14}C enriched algae, the second is with bacteria and the last one often with yeast.

Two isotopes can be combined in order to estimate the grazing impact and the selectivity on bacteria and phytoplankton cells or incorporation of specific compounds (Goulden and Place 1990). Phytoplankton is generally labelled with ^{14}C-bicarbonate, a tracer substance that can be added before the phytoplankton is placed in natural samples, or directly in the water (Landry 1994). In the dual conditions, radioisotopes labels are added to the natural assemblage, which is, incubated *in situ* and sub-sampled at intervals over a period of several hours (Harris *et al.* 2000). The Haney chamber is a well-used tool and permits the quick homogenisation of a sample of labelled algae (usually from a pure culture) within the natural community (Haney 1971).

The incubation time needs to be chosen carefully: it should be less than the time needed for intestinal transit, so that the tracer is not lost by defecation (Downing and Rigler 1984) and this period varies with the temperature, the type and concentration of food, the animal species and their size, and the characteristic of the incubation chamber.

1.4.3.2.Direct methods to measure assemblage grazing rates

a) *Community manipulations*

The community manipulation can be ever done by size fractionation or dilution.
The foundation of the size fractionation approach is that filters with different pore sizes are used to separate the zooplankton from its prey. Although this method is simple, evidence suggests that predators and preys may not be, unambiguously, separated by filters (Fuhrman and McManus 1984, Goldman and Caron 1985) and that the fractionation step may cause cell damage and media enrichment with dissolved organics (e.g. Fuhrman and Bell 1985, *in* Landry 1994).

As the dilution technique was used in this study, a more detailed description of it is presented hereafter.

b) An example of community manipulation: the Landry and Hassett's technique (1982)

Advantages and disadvantages of the method

The dilution technique estimates the specific growth and mortality rates from observed differences in their rates of population growth in a series of incubated diluted and undiluted seawater samples (Landry and Hassett 1982). The main advantage of the dilution approach is that it provides growth and grazing mortality estimates for all photosynthetic organisms in a single experiment. Mean growth rate estimates can be determined by chlorophyll analysis, microscopy, HPLC or flow cytometry. The main disadvantage of the dilution technique is that it does not remove all the grazing impact of microzooplankton; hence, relative to the fractionation, longer incubations are required to observe a significant change in prey density (Landry 1994). The most significant assumption of the technique is that the grazing rate varies linearly with the dilution factor. However some corrections should be done in some cases; for example, if the dilution reduces the prey density to a "threshold" level causing reduced grazing effort, the uncorrected analysis will lead to an overestimate of grazing impact (Landry 1994).

The Landry and Hassett equation

In this study, microzooplankton grazing was determined from measurements of the apparent growth rate of phytoplankton that were made assuming the exponential growth equation of Landry and Hassett (1982).

To be correct this equation proposed by Landry and Hassett (1982) requires three assumptions regarding the interactions of nutrients, phytoplankton and microzooplankton:

First, the growth of individual phytoplankton is not directly affected by the presence or absence of other phytoplankton *per se*. The implication of this is that a reduction in the density of cells in natural seawater will not directly cause a change in the growth rate of the remaining cells. Secondly, the probability of a phytoplankton cell being consumed is a direct function of the rate of encounter of consumers with prey cells. This implies that consumers are not food-satiated at natural prey densities and that the number of prey ingested by a given consumer is linearly related to prey density. Thirdly, they assumed that the change in the density of phytoplankton, P, over some time, t, can be represented appropriately by the exponential equation:

$$P_t = P_0 e^{(k-g)t}$$

where k and g are the instantaneous coefficients of population growth and grazing mortality; P_t and P_0 are the initial and final concentration of chlorophyll *a* over some time (t).

The rates of phytoplankton growth and grazing mortality can be inferred from the observed changes in population density following incubations of different dilutions of populations in natural seawater. Given a dilution series of unfiltered seawater of 10 %, 40 % and 70 %, the equations describing the changes in phytoplankton over time are:

Percentage of sea water		or
100%	$P_t = P_0 e^{(k-g)t}$	$1/t \ln (P_t/P_o) = k-1.0g$
70%	$P_t = P_0 e^{(k-0.70g)t}$	$1/t \ln (P_t/P_o) = k-0.70g$
40%	$P_t = P_0 e^{(k-0.50g)t}$	$1/t \ln (P_t/P_o) = k-0.40g$
10%	$P_t = P_0 e^{(k-0.10g)t}$	$1/t \ln (P_t/P_o) = k-0.10g$

The observed rate of change in the phytoplankton density at the different dilutions is linearly related to dilution factor; the negative slope of this relationship is the grazing coefficient g; the Y-axis intercept is the phytoplankton growth rate, k (see Fig. 1.2.).
Landry and Hassett argue that the observed rates of change of phytoplankton density at any two dilutions levels will yield two equations with two unknowns that can be solved explicitly for g and k. Linear regression analysis will provide estimates of the confidence limits for the coefficients.

1.5.The pigments as biomarkers

Pigments are increasingly used to characterize the algal community (Jeffrey et al. 1997). The ubiquitous chlorophyll *a* and its derivatives are very widely used to assess the phytoplankton biomass (Millie *et al.* 1993). Some of the pigments that can be separated, identified and quantified on an HPLC and relate to a specific algal class, are termed "marker pigments".

Some of these marker pigments are found exclusively in one algal class (peridinin found in dinoflagellates) while others are the principal pigments of one class but are also found in other classes (e.g. fucoxanthin in diatoms and others). The presence of these markers can be used as a guide to phytoplankton composition, as well as its biomass.

The advantages of estimating phytoplankton biomass and composition based on HPLC analysis of marker pigments are well know by its numerous applications in marine and estuarine systems (Millie et al. 1993) and, increasingly, in fresh waters. The development of advanced algorithms for calculating the contribution of algal classes, among which the CHEMTAX software (Mackey et al. 1996), are widely used and provides an additional benefit of the pigment approach. Data on marker pigments: chlorophyll *a* ratios in marine and fresh waters have been accumulating (Jeffrey et al. 1997, Schlüter et al. 2000, Lewellyn & Gibb 2000, Higgins *et al.* 2000, Descy et al. 2000) and several experimental studies have addressed their variations according to light and nutrients, in different classes (Goericke & Montoya 1998, Nicklisch & Woitke 1999). Automated HPLC analysis of extracts enables comparatively quick analyses and processing of many samples, thereby providing better spatial and temporal resolution than classic microscope techniques in large and complex aquatic systems (Fietz & Nicklisch 2004). An additional advantage demonstrated in several studies, since the introduction of reverse-phase HPLC analysis for phytoplankton surveys (Mantoura & Llewellyn 1983), is the detection of small algae, which can be overlooked by microscope examinations for different reasons (e.g. Gieskes & Kraay 1983). Undoubtedly, the pigment approach has become widely accepted as a superior technique for many applications, in particular as the method of choice to look at phytoplankton dynamics at the class level with a minimum of errors (provided that the pigment ratio variation is taken into account).

In this study the pigment concentration was used to estimate the grazing and growth rate by measuring the pigment concentration at the beginning and at the end of the incubation.

1.6. Dial vertical migration in algae

Diel vertical migration (DVM) has been identified as a competitive strategy for phytoplankton under conditions where light and nutrient are spatially separated (Ganf and Oliver 1982). Coordination of this behaviour with the necessary physiological adaptations for dark nutrient assimilation providing access to nutrients below the photic zone, may confer an adaptive advantage over co-existing non-migratory or non-motile phytoplankton such as diatoms.

Field data (Parker 2002) suggest that nitrogen and humic substances contained in the Huon River may affect DVM of phytoplankton. Humic substances affect the migration by reducing the total amount of photosynthetically active irradiation and may change the nitrogenous nutrition of phytoplankton by forming an alternative organic nitrogen supply (Granéli *et al.* 1985).

The Huon Estuary Study (2000) has study the vertical dynamics of *G. catenatum* by using a profiling system. This one gives a fluorescence profile on several days (5) and clearly demonstrates the DVM of *G. catenatum* in the Huon (as shown on Fig. 1.3.).
In this study, an attempt to simulate the DVM of *G. catenatum*, under non-nutrient gradient was done. The aim was to evaluate the impact of the zooplankton vertical migration on the DVM of *G. catenatum;* the only influence being the migrating zooplankton present in the water column.

1.7.Organization and aim of the thesis

The major goals of this study were to combine the training in modern, well-used techniques with the development of an appropriate research philosophy necessary to elucidate the understanding of current scientific problems. This experience introduced me to the organization of work within a governmental institution.

The first experiment was chosen with an aim to get some knowledge on an actual, major scientific question and to get some experience in using modern techniques. It is devoted to a series of field experiments and tries to evaluate the importance of microzooplankton grazing in a 10 m water column. In fact, some studies have shown that grazing could represent a loss of up to ~100% of the phytoplankton daily production (Verity *et al.* 1996) resulting in increased attention by researchers on the possible top-down control by the zooplankton on the phytoplankton (e.g. Fileman and Burkill 2001). The hypothesis tested in this study is that this grazing may vary with depth and that the advantage of motile phytoplankton species may be found in its capacity to avoid the zooplankton by migrating in the deep-layer while microzooplankton is feeding in the surface waters. We want to know if the position of a phytoplankton cell in the water column made a difference to survival.

The technique used to measure the grazing rate is the Landry and Hassett technique (1982): based on dilution of natural samples, it uses the pigment concentrations given by the HPLC (High Pressure Liquid Chromatography) to make a linear regression and to determine a growth rate (k) and a grazing rate (g). Until now, this remains the most reasonable single method for measuring the ingestion rate of whole microzooplankton communities because of the minimal manipulation of protozoan populations (Landry 1994).

In this study, the samplings were always made at the same site (Port Huon), and five different depths were sampled in order to establish a vertical profile of the grazing pressure in a 10 m water column. The five first experiments were done at midnight, in order to sample the migrating zooplankton, with a frequency of one per week (as the method is quite laborious). In addition, two day-time experiments were done, so that a comparison with the night-time data was possible.

The second part was a total laboratory experiment. Cultures of *G. catenatum* have taken approximatevely 3 months to prepare sufficient cells to fill in six water columns, each one containing approximately 5 l. Each column contained a stainless-steel cage, three of these containing calanoid copepods (experimental columns) and three others without zooplankton (controls). Cages were under the control of small motors (one for each column) and moved vertically in the columns, on a diurnal cycle (12:12 light:dark cycle). In order to know where the phytoplankton was located during the night- and day-time, samples were analysed with a fluorometer (Turner designs fluorometer©). A Repeated Measures MANOVA and a Generalized Linear Mixed Model was done on the data obtained.

In order to form a bloom, a species must have some competitive advantage over other species. For example, diatoms are more efficient in growing at low light level than most of the other species of phytoplankton. The environmental factors that encourage dinoflagellate blooms are not fully resolved. Of particular interest to phytoplankton ecologists is the possible competitive advantage of DVM. It is usually believed that the diurnal vertical migration allows the phytoplankton to reach nutrient-rich deeper layers, but this has not been proved to be the major factor permitting bloom formation. DVM is an inducible phenomenon; in some

species it has been correlated with the absence of nitrogen but it could also be a response to the presence of predators. Undergoing DVM to reduce predation has been shown to be an effective strategy in some zooplankton species and may be one of the reasons why dinoflagellates sometime have higher net growth rates than diatoms.

For the sake of clarity, the results, discussion and conclusion of these two experiments are presented separately. Future prospects to improve the knowledge of these concepts are included in conclusion.

Chapter 2: Materials and methods

2.1. Study context

The Huon estuary (see Fig. 2.1.), 60 km from the Tasmanian capital, Hobart, is situated between the latitude 42° 45'S and 43°45'S, in a maritime climate with north-westerly prevailing wind which generates variable and cool temperate conditions.

The estuarine zone extends for approximately 38 km, from Ranelagh to Huon Island, draining a catchment that includes areas of pristine wilderness and agriculture.

The Huon River estuary is a typical drowned river valley, and drains an area of approximately 3900 km^2 with monthly average flows ranging from 30-40 $m^3.s^{-1}$ (January-March) to 125-130 $m^3.s^{-1}$ (July-August) (CSIRO-Huon Estuary Study Team 2000).

The average maximal air temperature ranges from 11°C in July to 22°C in January and February. The average annual rainfall varies from 2000 mm in the west of the catchment to 800 mm in the east. Rainfall is relatively homogeneous throughout the year with maxima between July and October.

From a geological point of view, the west of the catchment presents some precambrian and cambrian rocks, typical of the south-west of Tasmania. In the central and eastern zones of the catchment, younger Permo-Triassic sediments can be observed, and in a few local areas, some Tertiary basalts are found. The soil ranges from acid to slightly alkaline. The nutrient concentration in the soil varies from low to medium for the total nitrogen and from low to high for the total phosphorus (Grant et al. 1995).

Estuaries are regions of the coastal ocean where salinity variations in space are so large that they determine the mean circulation. Compared to the flow in the direction of the estuary axis, cross-channel motion is very restricted, and the estuarine circulation is well described by a two-dimensional current structure.

An estuary is defined as a semi-enclosed coastal body of water having free connection to the open sea at least intermittently, and within which the salinity is measurably different from the salinity in the adjacent open sea. Estuaries can be grouped into classes, according to their circulation properties and the associated steady state salinity distribution. The most important estuary types are:

- Salt wedge estuary
- Highly stratified estuary
- Slightly stratified estuary
- Vertically stratified estuary
- Inverse estuary
- Intermittent estuary

The ratio of freshwater input to seawater mixed in by the tides determines the estuary type. One way of quantifying this is by comparing the volume *R* of freshwater that enters from the river during one tidal period, with the volume *V* of water brought into the estuary by the tide and removed over each tidal cycle. *R* is sometimes called the river volume, while *V* is known as the tidal volume. It is important to note that it is only the ratio *R*-*V* that determines the estuary type, not the absolute values of *R* or *V*.

The Huon River is a significant source of freshwater where it enters the head of the Huon Estuary at Huonville. Saline water enters the Channel from the open ocean and propagates up the estuary creating a classic salt wedge type estuary. These estuaries are characterised by high stratification and a stable water column; the water column only becoming well mixed during times of high river flow when the salt wedge is pushed back downstream.

Both the rapid flushing of the upper estuary and the very low concentration of suspended particulate matter limit the geochemical processes in the lower salinity zone. In contrast, primary production in the lower estuary affects nutrient concentration appreciably, and this influence would be doubtless extended to other trace solutes.

The main sources of chemical substances in catchment streams are: (i) weathering of rock to release dissolved minerals; (ii) leaching of soils and breakdown of plant material to increase both dissolved organic and inorganic substances; and (iii) surface runoff to sweep in suspended solids from land surfaces. These natural pathways contribute to the chemical load of freshwaters discharging to an estuary. Water quality in estuaries is strongly influenced by the material load delivered in surface runoff, but it can also be affected by direct inputs from ground waters. Direct atmospheric deposition to the estuary surface, by way of rainfall or fallout of dusts, is another natural input; but in Australia it is usually a minor contribution (CSIRO-Huon Estuary Study Team 2000).

The approximate dimensions of the Huon Estuary, below Ranelagh are shown in the following table:

PROPERTY	VALUE
Length	39.1 km
Width at mouth	4.5 km
Surface area	77.4 km
Volume	1.38 km^3

Port Huon (see Fig. 2.1.) is situated in the lower half of the estuary, 21 km north of Huon Island and has a depth of 10 m. This sample site has been chosen because it present regular *G. catenatum* blooms and it gives access to a 10 m deep water column, without the use of a boat, making the night-time sampling logistically easier. It was hoped the experiment would coincide with a summer-autumn bloom of *G. catenatum* although the abundance of this species at this location turned out to be quite variable.

Part 1 : Does grazing pressure vary with depth?

1.1. General

The samplings were made between the 2nd of March and the 29th of March 2005 with a frequency of one experiment per week, as the method is time-consuming and labour intensive (Landry and Hassett 1982). Additionally, day-time experiments were done on the 21st of April (surface and bottom).

Water samples were collected with 10 l Niskin bottles and passed through a 200 µm mesh to remove the large zooplankton. The water was stored in 10 l carboys; these bottles were kept in the dark and at low temperature until the water was put in the 50 l carboys in the laboratory.

1.2. Chemical variables

For each experiment, nutrient sample were taken, to determine whether the phytoplankton were nutrient limited during the incubation. Samples were taken at T_0 from the seawater, from the seawater that was used for the diluent and from the diluent itself. At T_{24}, samples were taken from the 100% seawater bottle and from the 100% diluent bottle.

Nitrate, nitrite, silicate and phosphate analysis was performed using the Quick Chem method on a LACHAT-instrument. The nutrient analysis methods used for each experiment were the following:

- Nitrate and/or nitrite: Quik Chem Method 31-107-04-1-A
  Determination of Nitrate and/or Nitrite in Brackish or Seawater by Flow Injection Analysis

- Silicate: Quik Chem Method 31-114-27-1-D
  Determination of Silicate in Brackish or Seawater by Flow Injection Analysis

- OrthoPhosphate: Quik Chem Method 31-115-01-1-G
  Determination of OrthoPhosphate in Brackish or Seawater by Flow Injection Analysis

More details on these methods are available on “www.lachatinstruments.com”.
The samples for silicate analysis were put in the fridge and not in the freezer as it is generally recognized that silicates polymerise during freezing.

The flow injection analysis uses a peristaltic pump to draw the sample from the sampler into the injection valve. Simultaneously, reagents are continuously pumped through the system.

The samples are then loaded in the sample loop, a chemical reaction that produces a colour takes place. The intensity of the colour is proportional to the concentration of the element being analysed for. The unknown concentrations are calculated from a linear regression of the colour of high precision standards versus known concentrations.

1.3. Physical variables

For each experiment physical variables were determined using a Sea-Bird SBE19plus instrument. This CTD is a high precision limnological and oceanographic tool. The CTD is made up of a set of small probes attached to a metal "cage". It measures a series of parameters including: temperature, salinity (calculated from conductivity), fluorescence, dissolved oxygen, pressure (calibrated to give depth) during a single cast. Back at the laboratory, the results are downloaded and processed on a computer using the program Seaterm, Seasave Win 32 and SBE DataProcessing-Win 32.

For more information see: http://www.seabird.com/products/spec_sheets/19plusdata.htm

1.4. Grazing experiments

Experiments to determine grazing rates were based on the dilution method of Landry and Hassett (1982). In this study, five experiments were conducted in order to sample the whole water column. One depth was analysed each week and the grazing impact was estimated at five different depths (1 m, 2.5m, 4 m, 7 m and 10 m). All the samplings were made at midnight in an effort to measure the impact of vertically migrating zooplankton. The incubations, in the water at the corresponding depth, started as soon as the dilution were prepared and terminated after 24 hours (midnight, the day after).

The material used for each experiment is given in Fig. 2.2. For each experiment 50 l of water were collected by 10 l Niskin bottles and screened through a 200 µm mesh to remove large zooplankton. Twenty litres of seawater were filtered through a Supor DCF™ filter (0.2 µm) (carboy 2). The other thirty litres were kept in the 50 l carboy (carboy 1).
Eighteen 2-litre polycarbonate bottles were previously rinsed in Milli-Q water, soaked overnight in 10 % HCl, rinsed again several times with Milli-Q, then soaked for 4-5 hours in warm Micro detergent and finally rinsed repeatedly in Milli-Q water. MilliQ water is here defined as water having been distilled and then deionised. Each of the 2 l bottles had their volume determined as accurately as possible before the experiments. A P-touch label was put on each bottle in order to have the most exact ratio diluent:seawater. When filling the bottles, silicone tubing was used in order to transfer the plankton as gently as possible. The seawater was then added to the bottles which needed it (100 % SW, 70 % SW, 40 % SW and 10 % SW) and they were filled to the top in order to avoid air bubbles (same method as the one used to fill in oxygen bottles). While making the different dilutions, the bottles are always kept in the dark.

The incubation bottles were put in cages and these were hung in the water at the corresponding depth (surface, 2.5 m, 4 m, 7 m and on the bottom). After ~24 hours the

bottles were taken out and filtered through a GF/F filter (47 mm, pore size ~0.7 µm), one dilution at a time (i.e. 100 % seawaters, then the 70 % seawaters, the 40 % seawaters and finally the 10 % seawaters and the diluent). The filters, placed in cryovials (Nalgene®), were stored in liquid nitrogen until analysis. Between the experiments, the equipment (incubation bottles and silicone tubing) was soaked overnight four times in Milli-Q water.

In order to augment the results obtained with the HPLC, three microzooplankton samples of 500 ml were taken at T_0 and T_{24} from the 100 % seawater. The microzooplankton was then preserved in 1 % of Lugol until counting.

1.5. Pigment analysis

In order to describe the diversity of microalgae, the pigments on the filters were extracted and analysed by HPLC (High Performance Liquid Chromatography). The HPLC allows a great range of chlorophylls and carotenoids to be measured rapidly (Jeffrey et al. 1997).

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column, where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high-pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. The separated pigments pass through the photodiode array, their absorption is determined across a range of wavelengths and this information is digitally collected and stored.

To extract the pigments, the filters cut into small pieces, were covered with 100 % acetone, vortexed and sonicated in an ice water bath for 15 minutes. After storage of at least 18 hours at 4°C, 0.2 ml MQ water was added to each tube (to bring the solvent to a ~ 90:10 acetone:water ratio) and the filters in solvent were sonicated again for 15 minutes in an ice-water bath. The content of each tube was then transferred to a Biorad® column holding a GF/F filter to remove the filter paper. The tubes were rinsed twice with 90:10 acetone-MQ water and the centrifuge tubes containing the Biorad® column were centrifuged for another 5 minutes (2500 rpm). The filtrate was then stored on ice and in the darkness until just prior to analysis. Before HPLC analysis, the extract was filtered through a 25 mm PTFE syringe filter with a pore size of 0.20 µm (Advantec MSF Inc.).

Pigment samples were analysed for pigment concentration and composition by high performance liquid chromatography with Waters® instrumentation (a Waters 996 Photodiode Array Detector, a Waters 600 Controller, and a Waters 717plus Autosampler). The HPLC system used an SGE 250*4.6 mm SS Exsil ODS (octodecyl silica) 5 µm column.
Pigments were eluted over a 30 minutes period with a flow rate of 1 ml min^{-1}. The gradient used was the follow (Wright *et al.* 1991):

1. 80:20 (v/v) methanol: ammonium acetate buffer (0.5M, pH of 7.2)
2. 90:10 (v/v) acetonitrile: MilliQ water
3. 100% ethyl acetate

Each solvent was pre-filtered through a Millipore HVLP 0.45µm filter.

The separated pigments were detected at 436 nm and identified against standard spectra using Empower™ software. Concentrations of the pigments were determined from standard (Sigma and purified pigments obtained from algal cultures).

Pigment concentrations of all experiments were processed, separately for each experiment, with the CHEMTAX software (CSIRO Marine Laboratories, Hobart, Australia; Mackey *et al*. 1996) using an input ratio matrix derived from Higgins et al. (submitted). This processing allowed estimating chlorophyll *a* biomass of Chrysophytes, Cryptophytes, diatoms, Haptophytes, Prasinophytes and Dinoflagellates.

1.6. Microzooplankton and phytoplankton counting

In order to complete the data obtained with the HPLC, three samples of 500 ml, both for T_0 and T_{24}, were preserved, in lugol, from each experiment. From the six samples of each experiment, two of the T_0 and two of the T_{24} were used for the counting, the third sample being a back up.

The T_0 and T_{24} Lugol preserved samples were first, transferred to 1-litre measuring cylinders and the samples were allowed to settle for 24 hours. After the volume had been recorded (V_1), 90 % of the volume was siphoned off and the remaining was transferred to a 100 ml measuring cylinder and left to settle again for 24 hours. Finally, 90 % of the volume was siphoned off, the final volume was recorded (V_2) and thoroughly mixed before a 1 ml aliquot was taken and put in a Sedgwick Rafter Cell and examined under an inverted microscope (Olympus® IX71).

Each Sedgwick Rafter chamber is composed of 1000 squares each containing 1 µl. For microzooplankton the entire slide was counted on a 10x scale. For microplankton (cells generally larger than 20 µm diameter), 100 squares or 10 % of the counting chamber was scanned on the 10x scale (except in cases where there were dense blooms of one or more microplankton species, when at least one column of 20 squares was scanned).
The identification guide used was the "Marine Phytoplankton Southern Tasmania" from Jameson and Hallegraeff (2000).

The following formula was used to determine the number of cells per litre in the samples:

Cells per litre = cell "species" count* (1000/ number of squares counted)*(V_2*1000/V_1)

Part 2 : Does the presence of zooplankton stimulate the vertical migration of *Gymnodinium catenatum* ?

2.1. Culture of Gymnodinium catenatum

The *G. catenatum* culture started on the 26 of February 2005 in 75 ml flasks maintained at 20°C±0.5°C on a 12:12 light:dark cycle. Two strains of *G. catenatum* were obtained from the CSIRO Collection of Living Microalgae (CSIRO Microalgae Research Centre) GCDE08, (isolated by S. Blackburn, 13/01/1986) and GCHU11 (isolated by S. Blackburn, 06/06/1986). These strains have been maintained in seawater medium with nutrients, soil extract and stock solution (nitrate, phosphate, vitamins, PII metals and selenium). Cultures were transferred approximately every 8 days to maintain cells in the exponential phase of growth. The transfers were always made under aseptic conditions, in laminar flow hood supplied with 0.2 micron filtered air.

After approximately two and a half months, enough culture (~60 l) had been prepared to fill the columns and to start the experiment.

2.2. Experiment set-up and sampling

In this experiment, we used diluted *G. catenatum* cultures and zooplankton in six 1 m-high transparent Perspex cylinders (diameter: 0.10 m). The clean columns were first filled in with seawater and a vertical gradient of light was created by illuminating the tops of the columns and putting black plastic around the lower part of each column. After the columns had been scrubbed with hot water and thoroughly rinsed with Milli-Q, they were filled with 30% phytoplankton (1260 ml for four of the columns and 1470 for two others because they were a little bit larger) and 70% of filtered seawater (2940 ml for the first one and 3430 ml for the second one).

G. catenatum was allowed to acclimate to the new conditions (columns) without zooplankton for 24 hours. After this acclimation period, the empty cages were put in the columns. Fluorescence measurements were done during 12 hours, in order to see if the migrating, empty cages had an influence on the DVM. The day after, a net (200 µm mesh) was sewed around each cage and the calanoid copepods were enclosed in three of them. Three other cages served as a control.

Samplings were taken at top and bottom of each column both at noon and at midnight during 216 hours, by using a 50 ml syringe and a 1-meter length, of 1 mm diameter, tubing.
In order to see if the phytoplankton were moving between noon and midnight, samples were made every two hours during 50 hours.

All samples were analysed by using a Turner Designs model 10AU fluorometer© and samples were preserved in Lugol as a back up. Because of this regular sampling, a mix of culture and medium (ratio calculated to maintain the same concentration during all the experiment) was added to refill in the columns and so to keep the same gradient of light available through the water columns. Irradiance was measured with a Licor QSL100.

Chapter 3: Results and discussion

Part 1 : Does grazing pressure vary with depth?

For the discussion of the grazing experiments, the interpretation should be done with caution, in fact the night-time experiments are done in March, whereas the day-time experiments were done in April, and so, there is a variability due to the time and not only with depth. Moreover, the day-time experiments were done at only two depths (surface and bottom) whereas night-time samples were done at five different depths.

3.1 : Physics

Observations of salinity, temperature, fluorescence and dissolved oxygen were made with a CTD (Conductivity-Temperature-Depth) before each experiment. Vertical profiles obtained for each experiment are presented on Fig. 3.1.

The water column stratification depends of the salinity and temperature variation throughout the water column.

As the experiments were done during the night, the epilimion generally presents lower temperature than the metalimnion. The surface temperatures ranged from 11 to 14°C and the thermocline was situated approximately at 50 cm for all the experiments, presenting a stratified water column and reaching temperature up to 16.5°C.
An exception to this pattern was observed on week 2 (2.5 m) when the water column was isothermal (homogenous) (despite the calm weather with low wind speed present during the sampling) and an average temperature of 14°C was observed.

The salinity presented a typical profile of a classic salt wedge estuary:

In a salt wedge estuary, the river volume *R* is very much larger than the tidal volume *V*. The fresh water flows out over the seawater in a thin layer. All mixing is restricted to the thin transition layer between the fresh water at the top and the “wedge” of salt water underneath. The vertical salinity profiles therefore shows zero salinity at the surface and oceanic salinity near the bottom.
During all the experiments the salinity profiles were quite constant, presenting value of 34.8 PSU (practical salinity units ~ parts per thousand) at the bottom of the water column and zero at the surface (week 1, for example). The salinity increased suddenly in the first meter (1 m=34.6 PSU) of the water column to reach its maximal value, with some profiles presenting a more linear increase (week 4, where 34.6 PSU is reached at 7.5 m).

The oxygen profiles were very similar during all the experiments. The oxygen concentrations reached values of 3.2 to 6.5 mg/l at the bottom. In the surface, the oxygen concentration range went from 6.7 to 9.5 mg/l. A metalimnetic minimum was observed at 1 m and was probably due to the rapid decomposition of organic matter resulting from higher temperature than in the deeper water layers. The minimum oxygen value reached during these experiments was of 2.8 mg/l and was observed during the day-time experiments, probably resulting of higher temperature. The entire water column was usually well aerated.

The fluorescence profiles showed always an increase in the first 50 cm of the water column but this rise was much less marked during the day-time. In fact, the day-time profile showed a homogeneous profile of fluorescence while during the night-time experiment there was always a certain stratification of the fluorescence. Excepting the first and the last experiment, the night-time data (within the first meter) reached rapidly value of 4 FU (Fluorescence Unit). After this, the fluorescence decreased, presenting diverse profiles over the 5 weeks of sampling. Several patterns of vertical stratification were observed: (i) a homogeneous profile below 50 cm (week 2), (ii) another increase at 4.5 m (week 3) or (iii) a decrease in a homogeneous way down to 6 m then a rapid decrease (reaching value of 1.5 FU) (week 4). The data obtained for the last experiment (week 5) presented a slow increase throughout the entire water column; the phytoplankton seemed to be mostly there, at the bottom of the column during the night.

3.2: Chemistry

Nutrient analyses were done on each experiment and the results are plotted in Fig. 3.2. Nitrate seems to be a potential limiting nutrient with concentration below detection limit between 4 and 7 meters.

Silicate in an estuary is dominated by land sources. The silicate is mostly used to make the silicate walls by diatoms and silica-scaled Chrysophytes, and is very rarely limiting. It presented a maximum of 11.5 µM at 7 m and a minimum of 5.8 µM at 10 m, both observed in the T_{24} samples. During the day-time experiments, the silicate concentration presented similar value as during the night-time (average of 8.2 µM during the night-time and 8.01 µM during the day-time (T_{24})).

The 100% seawater samples in this study presented a general phosphorus (P) decrease at 4 m reaching a value of 0.3 µM, the surface concentration was of 0.5 µM (T_{24}). The P concentrations were generally slightly decreasing between T_0 and T_{24}, exception made for the sampling done at 7 m where the concentration increased from 0.345 µM at T_0 to 0.807 µM at T_{24}. The experiments done in April presented slightly higher concentration (0.539 µM and 0.563 µM for the surface and bottom waters-T_{24}) than those done in March.
The different sources of phosphate are the organic matter biodegradation, enzyme hydrolysis and zooplankton excretion.

Nitrate is generally the dominating form of dissolved nitrogen during the winter. During spring, autumn and summer, nitrite can become dominant as nitrate is strongly removed by biological activities and as the nitrate reserve stays in the bottom layers.
In this study, NO_x (nitrate + nitrite) concentration showed low values (average of 0.17 µM for nitrate, and 0.103 µM for nitrite in March (T_{24})), similar to the one obtained by the HES,

which showed high concentration (4-5 µm to 20 µM) during winter, while summer values were low (October-April). Some nutrients could have been added to the night-time experiments to avoid possible effects of nutrient limitation of algal growth and ensure similar conditions at all depths and dilutions. Although in some experiences, nutrients are added to dilution bottles to prevent them from becoming depleted in the less diluted treatments, both Landry and Hassett (1982) and Gifford (1988) reported that such additions could damage delicate microzooplankton. Moreover, adding nutrient result in changing the conditions that are observed in natural samples and the growth rate may be increased by this addition. The NO_x vertical profile showed, like phosphate concentration, a minimum for both nutrients at 4 m (below detection limit for nitrate and 0.049 µM for nitrite).
Higher values of NOx (0.945 µM of nitrate and 0.541 µM of nitrite in the surface-T_{24}) were observed during the day-time experiments.
Nitrate is the second assimilable form of mineral nitrogen than can be taken by phytoplankton up, after NH_4^+.

An other interesting observation is that, the ratio N:P was much higher in April, during the day-time experiments (ratio (average)=2.68), than in March during the night-time experiments (ratio (average)=0.38).
Microalgae assimilate N and P at Redfield ratio (16:1), therefore in the Huon Estuary P was in excess relative to N. The nitrate:phosphate ratio in marine waters has been used as an indication of nutrient limitation when it departs sharply from the Redfield ratio of 16:1.
In coastal waters, a range of effects causes departures from the classic assimilation and remineralisation pathway for nutrients found in pelagic waters. These include runoff from agricultural land, effluent from urban centres (treated sewage, storm water, etc.) and discharges from industrial activities (via effluents or aerosols). Biological pathways for nutrients in coastal waters are also different and more complicated. Exchange with sediments, macrophytes, wetlands and other coastal features all leave their imprint. Nevertheless, gross changes in the nitrate: phosphate ratio can still be useful in assessing pressures on microalgal production in coastal waters (Thompson 1998).

3.3: Biology

The results are here presented by comparing the night- and day –time data after having described them. The interest is focused on the following groups of plankton: dinoflagellates, diatoms, cryptophytes and grazers. The averages given here are those of the 100% seawater samples and the length of incubation is always mentioned.

a) HPLC

While microscopic examination reveals the most information about the taxonomic composition of phytoplankton assemblages, quantification of pigments can yield similar kinds of information with less manpower.

The pigment concentrations in this study were used to determine the differences in phytoplankton biomass and composition between T_0/T_{24}, depth and day/night.

HPLC pigments that relate specifically to an algal class are termed "marker pigments" (see Table 3.1). The presence of these markers can be used to determine the phytoplankton composition as well as its biomass. We have used chlorophyll *a* (measured by HPLC) as a measure of total phytoplankton biomass and the marker pigments to characterize the composition of the phytoplankton community. In total, seventeen pigments were identified.

An example of typical chromatogram obtained by the HPLC is represented on Fig. 3.3. The pigments are identified by their retention time and their shape.

The pigments we focused on range from the most broadly distributed chlorophyll *a* (in all taxon) to the alloxanthin, only find in Cryptophytes. Some pigments are intermediate in their distribution and hence in their usefulness for chemotaxonomic studies of phytoplankton (Everitt *et al.* 1990). Peridinin is, for example, found only in dinoflagellates, but not in all dinoflagellates (Jeffrey 1980); fucoxanthin is found ubiquitously in diatoms, but also in Chrysophytes and Prymnesiophytes, which are morphologically and ecologically quite distinct from diatoms.

Pigment	***Algal class***
Peridinin (Perid)	**Dinoflagellates**
Fucoxanthin (Fuco)	**Diatoms**
19'-hexanoyloxyfucoxanthin (Hex-fuco)	**Haptophytes**
Alloxanthin (Allo)	**Cryptophytes**
Prasinoxanthin (Pras)	**Prasinophytes**
Zeaxanthin (Zea)	**Cyanobacteria**

Table 3.1. Major marker pigments and the algal classes they represent.

After being analysed with CHEMTAX, the results were plotted as area graphs for each depth and for the day-/night-time experiments.
Fig. 3.4. presents an area graph using the average concentration of T_{24} for night- and day-time experiments. The average for T_0 and T_{24} are plotted on Fig. 3.5. for both night- and day-time results.

Night-time experiments

The pigment concentrations observed were well correlated to the different dilutions with a decrease in pigment concentration in the most diluted samples and little variation of the community was observed between T_0 and T_{24} (see Fig. 3.5.).

Based upon CHEMTAX, the phytoplankton community was dominated by dinoflagellates in the whole water column. Two maxima are observed in the water column for dinoflagellates: one at 4 m with an average value of 90.2% of total chlorophyll *a* (T_0) and another one at 10 m reaching 84.5% of total chlorophyll *a* (T_0).

In the Chesapeake Bay, estuary dinoflagellates were also reported as the dominant Class of phytoplankton during the summer (McManus and Ederington-Cantrell 1992). In the Chesapeake, however, diatoms were found to be to be an important component of the phytoplankton throughout the year. During sampling in March 2005, diatoms only represented up to 10.7% (T_{24}-surface) of total chlorophyll *a.* However, we will see that this community had increased in April 2005; during Spring 1997 diatoms were ~ 90% of the phytoplankton biomass (CSIRO-Huon Estuary Study 2000).

The second most abundant class was Cryptophytes, characterized by alloxanthin; they presented maximal value of 14.0% of total chlorophyll *a* at 2.5 m (T_{24}) (see Fig. 3.4). Prasinophytes were present at low concentrations in the whole water column and are mostly situated at 7 m, representing at this depth 5.8% of total chl *a*.

Day-time experiments

Surface waters presented a community with high diversity during the day-time experiments in April 2005: Chrysophytes, diatoms, Prasinophytes and Cryptophytes were present. During these experiments, the phytoplankton community was dominated by Cryptophytes.
Cryptophytes are, visibly, more situated at the bottom of the water column (86.0% of total chl a-T_0) than at the top (51.9% of total chl a at T_0) while diatoms represented up to 22.8% (T_0-surface) of total chlorophyll *a* and their biomass decreased with depth (7.8 % at 10 m).

The chlorophyll *a* concentration, during the day-time experiments, rose in the surface waters from 0.320 µg chl a/L at T_0 to 0.611 µg chl a/L at T_{24}, which means that the biomass almost doubled in 24 hours.

Comparison between day-/night-time experiments

Fig. 3.5. shows that the phytoplankton community composition, biomass and diversity changed between April and March 2005.

In March, the community was largely dominated by dinoflagellates whereas the Cryptophytes appeared to be the major group present in April.

Note (see Fig. 3.5.) that the general biomass was much lower in April compared to March.

b) Counting

Counting results are plotted on Fig. 3.6. and 3.7. for night- and day-time.

Cell count results for the day-/night-time experiments

Night-time experiments

Diatoms were dominated by the genera *Chaetoceros*, *Skeletonema*, *Pseudonitzschia* and *Proboscia*. In the first few meters, the concentration in diatoms was homogenous (average of 2,588 cells/l) and increased at 7 m, to reach a concentration of 23,247 cells/l at 10 m.
The numbers of diatoms cells always increased between T_0 and T_{24} at all depths and the maximal increase observed was of 30,043 cells/L in 24 hours at 7m.

The autotrophic dinoflagellate community was largely dominated by *Ceratium* (*C. tripos*, *C. furca* and *C. lineatum*) but *G. catenatum* and *Dinophysis* were also common. As for diatoms, *Ceratium* reached its highest concentration at 10 m depth (51,715 cells/L-T_{24}).
Between T_0 and T_{24}, the number of cells per litre stayed more constant for the dinoflagellates than for the diatoms.

Microzooplankton grazers included copepod nauplii, tintinnids, heterotrophic dinoflagellates (e.g. *Polykrikos*) and ciliates.

The numbers of ciliates were constant during the first few experiments and increased suddenly during the last two weeks of March, to end with a concentration of ~1,000 cells/L.
The numbers of *Polykrikos* reached important concentration during week 3 (4 m), starting with 45 cells/L at T_0, it ended with 179 cells/L at T_{24}. The week after, *Polykrikos* were still increasing in the water column: 1,717 cells/L were found at T_0 and 4,781 cells/L at T_{24}; after this period, *Polykrikos* numbers decreased. Jeong *et al.* (2001) investigated the growth coefficients of *Polykrikos kofoidii* when feeding on several species of red-tide and/or toxic dinolagellates; growth rates of *Polykrikos* on *G. catenatum* were very high and the grazing of these seemed to have sometimes considerable impact on prey populations.

The general grazer concentrations were very low from 0 to 4 m and maximal value was observed at 7 m. At this depth, all diatoms, dinoflagellates and grazers have increased between T_0 and T_{24}. Some decrease in the number of grazers between T_0 and T_{24} were observed (2.5 m and 10 m), a possible explanation may be that larger grazers had consumed smaller microzooplankton, or possible mis-identification of autotrophic taxa as heterotrophic, or vice versa.

In conclusion, all the organisms seemed to be mainly, and following the cell counts results, at the bottom of the water column during the night.

It is important to note that the scale value is quite different for grazers, diatoms and dinoflagellates mainly because of the differences in the cell volume between these three groups of organisms. The average values for T_{24} are of 2,158.3 ± 15,337.5 cells/l for the diatoms; 29,968.9 ± 21,932.8 cells/l for the dinoflagellates; and of 1,942.7 ± 2,706.1 cells/l for grazers.

Day-time experiments

The diatoms seemed to be homogeneously distributed in the water column of the Huon Estuary during the day-time in April 2005.
The counting results of diatoms in the surface samples showed that there was an increase of cell number (per litre) between T_0 and T_{24}, ranging value of 2,348 cells/L at T_0 to 5,440 cells/L at T_{24}, whereas the diatoms situated at the bottom decreased from 2,618 cells/L (T_O) to 1,600 cells/L (T_{24}).

For the dinoflagellates, the day-time experiments shows a great difference in cell numbers between the surface and the bottom of the column: 2,024 cells/L at the surface and 72 cells/L at the bottom are observed at T_0. Like the night-time experiments, the concentration of dinoflagellates varied little between T_0 and T_{24}.

The number of grazers in the surface layer was higher than at the bottom of the water column (T_0-surface: 625 cells/L and T_0-bottom: 136 cells/L). The surface waters were mainly dominated by heterotrophic dinoflagellates (375 cells/L-T_0) and ciliates (150 cells/L-T_0); ciliates (73 cells/L-T_0) and copepods (45 cells/L-T_0) were the dominant grazers at the bottom.

Comparison day/night-time experiments

The phytoplankton community was very different between the March- and the April-experiments, this resulting more than probably of a seasonal change in the conditions. This variability could have been avoided by doing both experiments during the same month.

While diatoms were mostly situated at the bottom during the night-time, they presented a homogeneous profile during the day-time. As for the diatoms, the dinoflagellates were mostly situated at the bottom during the night but presented a biomass concentrated in the surface layer during the day-time. Grazers presented a general higher biomass in the surface during the day-time, and conversely, most of the community of grazer was situated at the bottom during the night-time.

The general number of cells was higher during the night-time experiments than those obtained for the day-time experiments.

Both during night- and day-time experiments, the microzooplankton seemed to follow the phytoplankton, and especially dinoflagellates, up and down in the water column.

Comparison of cell count results with the HPLC results

In order to make the comparison easier, concentration of marker pigments for grazers (total phaeophytin a-like), diatoms (fucoxanthin) and dinoflagellates (peridinin) were plotted on the cell count histogram (Fig. 3.6. and 3.7.). Due to the absence of sufficient counting results for the Cryptophytes, they were not included in this comparison. The general variation of cell counts and pigment concentrations are similar. Some large differences were observed between the grazer cell counts and the concentration of total phaeophytin a-like, indicating that the concentrations of these chlorophyll *a* degradation products were not related to microheterotroph grazing rates.

Most of the HPLC results are recognizable in the cell counts, which, however, have the capacity to distinct the species present and so can bring some interesting information for the grazing measurements. The HPLC has the advantage of being able to analyse more samples in less time, it is more accurate and provides a result for taxa that are too small for enumeration or identification under the light microscope (such as many Cryptophytes and cyanobacteria). Thus the combination of both techniques is interesting because they compliment each other.

c) Grazing measurements

For the sake of clarity, we mainly focused for the grazing experiments on total chlorophyll *a*, peridinin, fucoxanthin and alloxanthin. The grazing rate, growth rate, net phytoplankton growth rate are systematically presented as follows: average ± standard error. Graphs representing the grazing rate, growth rate, ingestion rate and percentage of primary production grazed per day for all depths are plotted on Fig. 3.8. for the night-time experiments and on Fig. 3.9. for the day-time experiments. All the exact values are found in Table 3.2.

A typical result from a grazing rate experiment demonstrates a linear relationship between the dilutions and the apparent growth rates. Two important estimates can be made by using the relationship: the Y-axis intercept is the estimation of the phytoplankton growth rate in absence of any grazing (d^{-1}), and the second is the slope of the regression line, which corresponds to the grazing rate by microzooplankton on phytoplankton (d^{-1}). The equation naturally returns the grazing as a negative number and growth as a positive number. Often grazing rates are considered a loss term and are therefore expressed as a negative term. We report here the grazing rate as negative value but most of the more recent literature reports them as positive. The regression analysis provides standard errors for both measurements. The determination coefficient permits us to quantify the intensity of the relationship between the dilution factor and the apparent growth rate.

The potential percentage of primary production grazed (expressed as %) was measured by using the following formula:

$$100*(1-e^{(-g)})/(1-e^{(-\mu)})$$

where g is the grazing rate (d^{-1}) and μ the apparent growth rate (d^{-1}).

The ingestion rate (μg chl^{-1} d^{-1}) was estimated by using the following formula:

$IR = P_i * P_0$

where P_0 is the initial concentration of pigment (μg/L)
and P_i (μg/L) = $(1-e^{(-g)})*100$

Night-time experiments

The linear regression done on chlorophyll *a*, fucoxanthin, alloxanthin and peridinin are represented on Fig. 3.10.

The phytoplankton growth responses (see Fig. 3.10.) were linear with the fraction of unfiltered seawater for most dilution experiments. In some cases, however, particularly at low initial prey concentrations, the response was non-linear (e.g. night experiment, at the surface for the apparent growth rate based on chlorophyll *a*). Non-linearity has been reported at very low and high prey densities by Gallegos (1989).

For the results based on chlorophyll *a*, a positive grazing rate (0.099 ± 0.149 d^{-1}) and an excessive low growth rate (-0.18 ± 0.0959 d^{-1}) were observed for the first experiment. The first experiment resulted in a markedly different grazing rates than for all the other depths; this could be due to the fact that these cells were held 24 h at high (surface) light levels and so experienced pigment bleaching; thus, their growth in terms of pigment were very low.

The grazing rate, based on chlorophyll *a*, was maximum at 10 m depth (-0.623 ± 0.0880 d^{-1}) and minimum at 7 m (-0.38 ± 0.0880 d^{-1}).
McManus and Ederington-Cantrell (1992) found slightly lower grazing rates in Chesapeake Bay; phytoplankton grazing mortality (based on chlorophyll *a*) ranged there from <0 to 1.6 d^{-1} in the surface waters.

When using chlorophyll *a* as an indicator of the whole phytoplankton community, apparent phytoplankton growth rate ranged, at Port Huon in March 2005, from -0.18 ± 0.0959 d^{-1} in the surface to 0.593 ± 0.0567 d^{-1} at 10 m. Growth rates calculated by McManus and Cantrell (1992) ranged from 0 to 2.15 d^{-1}, but the majority of rates were <1 d^{-1}. Their results for a station situated in the mid-bay (equivalent to Port Huon) had chlorophyll *a* growth rates of 0.23 d^{-1} (average over all dates; SE = 0.05). The growth rates based on pigments other than chlorophyll *a*, as for the Huon Estuary, were higher. Fucoxanthin, for example, was the pigment with the highest growth rates in the Huon and reached growth rates in Chesapeake Bay of 2.91 d^{-1} in the upper bay (when, nutrients were added to the bottles).

Net phytoplankton growth rates (= grazing rate + growth rate) were always negative (ranging from –0.176 ± 0.284 d^{-1} at 7 m to –0.030 ± 0.105 d^{-1} at the bottom of the water column); this resulting more than probably of too low levels of light intensity to ensure the phytoplankton growth. However, net phytoplankton growth rate based on fucoxanthin (0.3 ± 1.10 d^{-1} at 0 m and 0.072 ± 0.209 d^{-1} at 7 m), and alloxanthin (0.394 ± 0.65 d^{-1} at 0 m), presented positive net growth rate during the night-time in March 2005.

The impact of grazing, based on chlorophyll *a*, during night-time seemed to be in the Huon, very important and represent, excepting the first experiment (52.1%), more than 103.8 % (10 m) of the primary production! The greatest value observed was of 175.3% of the primary production at 7 m! These results suggest that there was a strong top-down control by microzooplankton on phytoplankton, especially during the night.

The relatively important role of microzooplankton as primary consumers has been noted in several experiences (McManus and Fuhrman 1988, Gifford 1988). Because they can grow and multiply as rapidly as phytoplankton cells, protistan microherbivores derive considerable advantage over larger metazoans in their ability to exploit ephemeral changes in food availability (e.g. Miller *et al.* 1995). Their grazing pressure is thus better coupled to production processes relative to slow-responding metazoans.

The ingestion rate based on chlorophyll *a* increased sharply at 10 m to reach value of 4.28 µg chl a^{-1} d^{-1}.

A linear regression was done on peridinin vs. dilutions to evaluate the impact of grazing on dinoflagellates. The highest grazing rates on peridinin were observed at 2.5 m (–2.7 ± 0.31 d^{-1}) and the lowest in the surface waters (-1.3 ± 0.41 d^{-1}). These results show that grazing impact on dinoflagellates is quite high during the night, however, diatoms were even more heavily grazed. Dinoflagellates were grazed in a homogeneous way in the entire water column (average of –2.4 ± 0.60 d^{-1}), excepting the surface waters.

Growth rates of peridinin, but also of fucoxanthin and alloxanthin, were substantially greater than those of chlorophyll *a*. For example, growth rates based on fucoxanthin were 19 times greater than those of chlorophyll *a* at 7 m. Fucoxanthin growth rates exceeding chlorophyll *a* in such a proportion suggest that diatom population was growing rapidly, even when it was not the dominant biomass. Growth rates based on peridinin were 11 times greater than those of chlorophyll *a* at 7 m, indicating that dinoflagellates also were growing more rapidly than the phytoplankton as a whole. Excepted for the first experiment, the gross growth rate did not vary a lot in the water column for peridinin. At 2.5 m, the net growth rate calculated for peridinin showed its highest value of –0.293 ± 0.37 d^{-1}. The percentage of peridinin production grazed is homogeneous in the water column (average of 104.2%).

Estimates of grazing rates upon diatoms based on fucoxanthin, assuming that fucoxanthin is associated with diatoms (some microflagellate groups may also contain this pigment, though in lower abundance relative to chlorophyll *a*; Vesk and Jeffrey 1977, Wright and Jeffrey 1987), showed that diatoms were always grazed more rapidly than dinoflagellates. Grazing on diatoms was maximal at 2.5 m (-3.86 ± 0.248 d^{-1}) and the gross phytoplankton growth rate was maximal at 7 m (3.77 ± 0.113 d^{-1}). The percentage of fucoxanthin production grazed was relatively homogeneous with depth with an average of 99.8%. As demonstrated by the determination coefficients, the intensity of the relationship between the dilution factor and the apparent growth rates, based on fucoxanthin, were always high (at least 0.96 (2.5 m)), excepted (again!) for the first experiment (R^2=0.50).
Ingestion rates based on fucoxanthin reached a maximum value of 0.05 µg chl a^{-1} d^{-1} at the surface and was always lower than the ingestion rate based on peridinin. Fucoxanthin showed positive net growth rates at two different depths indicating that diatoms and possibly other fucoxanthin-containing algae had sufficient cell growth rates to compensate for grazing losses during night-time. Fucoxanthin was grazed faster than peridinin but also grew faster giving higher net growth rates for diatoms. Because the abundance of diatoms was low, the absolute amount ingested by microzooplankton was high relative to peridinin or chlorophyll *a*.

The fact that grazing was weaker on dinoflagellates, may explain some of the reasons of their important development at some period of the year. Jeong *et al.* (2001) have shown that *Polykrikos* was an important predator of dinoflagellates, moreover, we know, as shown by cell counts, that the biomass of *Polykrikos* was increasing in the Huon during the two last weeks of March 2005, this could explain the increase in grazing rate that was observed during the night-time experiments in March 2005.

The linear regression of alloxanthin (Cryptophyte) concentration versus percent seawater gave similar results as for peridinin excepted that positive net phytoplankton growth rates were observed in the surface (0.394 ± 0.65 d^{-1}).

Day-time experiments

The linear regression done on chlorophyll *a*, fucoxanthin, alloxanthin and peridinin are represented on Fig. 3.11. for the day-time experiments.

We found grazing and growth rate to be closely coupled within individual pigments: grazing and growth rates, based on total chlorophyll *a*, was greater at the surface, reaching value of -1.18 ± 0.141d^{-1} (g) and 1.25 ± 0.0906 d^{-1} (k), than at the bottom (-0.331 ± 0.0692 d^{-1} (g) and 0.35 ± 0.0446 d^{-1} (k)).

In the same way, the net phytoplankton growth rate, based on chlorophyll *a*, was lower at the bottom of the water column (0.018 ± 0.0824 d^{-1}) than at the surface (0.069 ± 0.167 d^{-1}). The percentage of primary production grazed was similar at both depths (95.5% at the bottom and 96.8% at the surface) and the ingestion rate, like the grazing and growth rates, was greater at the top (0.20 µg chl a^{-1} d^{-1}) than at the bottom (0.072 µg chl a^{-1} d^{-1}).

The linear regression done on peridinin vs. dilutions provided more information about specific grazing. The slope observed during the day-time experiments was equal to zero or positive, and so demonstrated that there was probably no grazing (or very little) on dinoflagellates.

Consistent with the night-time results, diatoms seemed to be more grazed than dinoflagellates. At the surface, diatoms were grazed at a rate of –2.05 ± 0.40 d^{-1} and Cryptophytes, which were the second most grazed group, at a rate of –0.23 ± 0.281 d^{-1}. As for grazing rates, gross phytoplankton growth rates were markedly greater for diatoms (2.09 ± 0.260 d^{-1}) than for Cryptophytes (0.30 ± 0.181 d^{-1}) at this depth. Results expressed in % of primary production grazed, show value of 99.3% of diatom production and of 80.0% of cryptophyte production in the surface waters.

Bottom waters presented similar values to those of the surface waters for grazing rates (-2.4 ± 0.40 d^{-1}) and gross growth rates (2.14 ± 0.257 d^{-1}) for diatoms. At the bottom, grazing (-0.3 ± 0.40 d^{-1}) and gross growth (0.44 ± 0.260 d^{-1}) rates for Cryptophytes were lower than at the surface. All marker pigments, excepted peridinin in the surface sample, showed positive values of net phytoplankton growth rate.

In conclusion, the percentage of fucoxanthin production grazed was always higher than for Cryptophytes and the difference was more marked at the bottom of the column (70.4% for Cryptophytes and 103.4% for diatoms). The determination coefficient (r^2) was extremely low at the bottom of the column for Cryptophytes and reached a value of 0.05.

Comparison day-/night-time experiments

A direct comparison of the grazing rates obtained during day- and night-time experiments extract the important information of these grazing rates results. The grazing rates were always higher during the night-time, for all the marker pigments analysed, excepting the grazing rate based on total chlorophyll *a*, which was greater during the day in the surface than during the night at the same depth (surface day-time: -1.18 ± 0.141 d^{-1} and surface night-time: 0.099 ± 0.149 d^{-1}). An important difference between the experiments done in March and in April 2005, is the difference in the grazing rates for dinoflagellates. Biomass and grazing rates based on peridinin were greater in March 2005 than in April 2005, where grazing on dinoflagellates was very restricted. The similar increase observed in the grazing pressure and the abundance of *Polykrikos* in the water column during March 2005, prove that these grazers are, as recognized by other studies (Jeong *et al.* 2001), important control of the dinoflagellate biomass. These organisms may help, in the development of a community dominated by Cryptophytes, by grazing on dinoflagellates.

The gross growth rates were greater during the night-time for alloxanthin and fucoxanthin, but greater during the day-time for peridinin and total chlorophyll *a* at the surface.

The net phytoplankton growth rate presented positive values during the day-time as the solar energy was present to ensure growth.

Difference of ingestion rates, between day- and night-time, was only observed for the dinoflagellates (ingestion rate more important during the night) and diatoms (ingestion rates greater during the day).

Part 2 : Does the presence of zooplankton stimulate the vertical migration of *Gymnodinium catenatum*?

2.1. Analysis of Phytoplankton Data

The most generally accepted method of analyzing multivariate data of the variety we have is by the use of Repeated Measures MANOVA (O'Brien 1985). In this case however we do not have sufficient replication in our samples to allow this. We can either use a Repeated Measures ANOVA of the differences between the surface scores and the bottom scores or fit a Generalized Linear Mixed Model (GLMM) (McCullagh and Nelder 1983) with the water columns as the random effects. The Muachly's test failed to detect sphericity in the data (p-value 0.135) and so, we are not able to fit a Repeated Measures ANOVA. However, Muachly's test is of low power and will often fails to detect sphericity that is present in the data. We also fitted a GLMM and identified a p-value of 0.0064 for the day-night term in the model suggesting there are day/night variations in the columns. A plot of the residuals against the fitted values from the Generalized Linear Mixed Model shows that the data are not normally distributed (Fig. 3.12.): this would tend to invalidate both the GLMM and the Repeated Measures ANOVA.

It is the opinion of the analyst that the sample size was too small to do any meaningful analysis; if the experiment were re-run with a larger sample size, say 24 columns, then Repeated Measures MANOVA can be used to analyse the results.

The statistical packages SPSS version 11.5 and R version 2.11 and Microsoft Excel 2000 were used in this analysis.

2.2. Data analysis

Despite the failure of statistical test, a discussion of the fluorescence results is worth.
Three experiment columns, with zooplankton (EC_2,EC_5,EC_6) and three control columns (CC_1,CC_3, CC_4) were used in this experiment.

Sampling started in the columns without treatment (no cages, no zooplankton).
During the 24 hours of sampling without treatment, the phytoplankton concentration observed at the surface of the columns were very low with, excepted one of the experiment columns (EC_2), where an average of 2-3 FU (Fluorescence Units) was measured. Little variation (maxima observed = 4 FU for EC_2) was observed between day-time and night-time in the surface. The bottoms of the columns presented a more important biomass; the greatest difference observed between day and night being of 14 FU for one of the control column (C_1).

After these 24 hours, the empty cages were all put in the columns in order to see if they would change, by themselves, something in the phytoplankton migration. One sampling at noon and one at midnight were done for this step, the control column-CC_1-was the only column showing a minor increase in biomass at the bottom during day- and night-time (increase of 0.5 FU).

For the presentation of the results with zooplankton in the cages of the experiment columns (see Fig. 3.13. and 3.14.), the column EC5 (experiment control) was excluded because of its very high FU value which obscures the variation of the other columns. Due to a technical problem with sampling the column EC_5 reached an apparent concentration of ~200 FU.

After 24 hours in presence of zooplankton, phytoplankton seemed to move mostly in CC_1 and EC_6. The fact that phytoplankton were migrating in CC_1 tends to reject our hypothesis, that the phytoplankton migration could be influenced by the presence/absence of zooplankton. Moreover, both surface and bottom waters seemed to increase their biomass during the night; this being the opposite of what we expected: the phytoplankton should be more in the surface during the day than during the night and in our case, the phytoplankton presented a general higher biomass at the bottom during day and night. This could be due to the fact that phytoplankton is not staying in the first 10 cm of the water column (where surface sampling were taken) to avoid a too high light intensity.

The difference in fluorescence between surface and bottom is plotted on Fig. 3.15. This graph shows that, after 24 hours, the phytoplankton seemed to move in the columns and the importance of this migration seems to decrease after ~96 hours. This could be the result of the zooplankton's death (as proved after examination under microscope at the end of the experiment) but unfortunately, the exact time of these deaths are not known.

The "two-hours sampling" showed that phytoplankton was moving vertically in the water column throughout the day and that it was increasing its biomass in the end of the afternoon (between 16 and 20 p.m. the first day, and between 14 and 16 p.m. the second), showing that phytoplankton is at its highest biomass at this time in the surface layer. The bottom scores were variable.

The phytoplankton seemed to have shown different dynamics between the various columns but the cells in the control columns were as variable, in their vertical migration behaviour, as the experiment columns.

Even if our hypothesis could not be demonstrated in this experiment, it was an experiment requiring a range of skills, introducing some aspects of experimental design and requiring the set-up of an experiment with all the knowledge linked to that.

Chapter 4: Conclusion

Part 1 : Does grazing pressure vary with depth?

In this study, our focus was on microzooplankton grazing; microzooplankton are able to select food, based on size and nutritional value (Anderson 1997), which coupled with a rapid population response to changes in food abundance, allow them to play a significant role in structuring plankton communities and determining the fate of phytoplankton production within the euphotic zone and its export from it (Strom and Strom 1996). Grazing by these small zooplankton serves to retain material in the upper water column because, unlike larger grazers, they do not produce sinking fecal pellets and thus contribute less to the vertical flux of material.

The dilution technique has been used to estimate grazing in this study and in a variety of environments, including tropical, temperate and artic regions (Landry *et al.* 1984, Burkhill *et al.* 1987, Gifford 1988). Gallegos (1989) extended its usefulness to eutrophic waters.

The results obtained in this study show that dinoflagellates and Cryptophytes were the major phytoplankton classes present in the Huon during autumn 2005. During the night-time, in March 2005, the dinoflagellates were mostly abundant at 10 m-depth while the Cryptophytes seemed to stay near the surface. Cryptophytes largely dominated the community during the day-time experiments in April 2005.

In common with a number of other studies (Burkhill *et al.* 1987, Gallegos 1989), we found that growth and grazing were closely coupled within individual pigments (phytoplankton taxa that were growing rapidly were also grazed rapidly).

As previous studies, we have shown microzooplankton grazing to be an important control for estuarine production (Capriulo and Carpenter 1983,Gifford 1988); we observed that zooplankton present during night-time, at 7 m depth could graze up to 175.3 % of chlorophyll *a* production during a 24 h period! Day- and night-time experiments were all done during 24 h, so that their included an approximatevely equal light period for phytoplankton growth.

Gross growth rates were affected by depth as the irradiance vary with this one.

The results obtained in this study show that grazing is a major control on phytoplankton during the night-time for all the pigment analysed excepted for grazing rates based on fucoxanthin, which showed that the grazing was more important for this pigment during the day-time and not during the night-time. We now know that grazing pressure seems to vary with depth and that microzooplankton seems to act as a top-down control mostly at the bottom of the water column during the night in the Huon Estuary. Moreover, this grazing may be selective and for example, *Polykrikos* may act as a strong top-down control on *G. catenatum* and other toxic dinoflagellates as proved by Jeong *et al.* (2001).

The population of this grazer have increased rapidly during March 2005 in the Huon Estuary and grazing rates measured on peridinin, followed this increase which means that *Polykrikos* was probably, in our study too, an important control of dinoflagellate community. Getting the population of dinoflagellates under control, to avoid bloom formation, may require the presence of specific grazer (e.g. *Polykrikos*) and this important control may even allow the transition to a community dominated by Cryptophytes, as in April 2005.

Two important conclusions have been here proven; the first one is that grazing is not equal with depth but that certain depths present higher grazing than others in the Huon Estuary. The implications of this, is that phytoplankton may try to minimize grazing by avoiding those depth of high pressure and so the mobility of dinoflagellates may be an advantage for their development at some period of the year in the Huon Estuary. The second important conclusion, is that grazing at certain depth varies with time of day (surface night vs. day comparison). On a short term basis we could expect to see behaviours in motile species to avoid depths (and time) of high grazing pressure, like diel vertical migration which, was observed for *G. catenatum* in the Huon by the Huon Estuary Study (2000).

In the Huon Estuary, grazing seems to act to retain material in the water column, as a mass of microzooplankton and their excretions, and probably results in higher production of copepods and other macrozooplankton that graze on microzooplankton.

However, in both conclusions, some allowance needs to be made for the fact that not all experiments were carried out at the same time.

Moreover, a wide range of growth and grazing rates were observed among taxa in this study, suggesting that net phytoplankton growth rates derive important components of their variability from both intrinsic variation in phytoplankton growth rates and selectivity of grazers among taxa.

It seems likely that the experimental design could be improved by reducing the time between measurements. The next step in this study could be to do the day-time experiments closer in time to their equivalent depth during the night-time experiments and conduct all the sampling as closely as possible in time. We could, for example, do fewer dilutions and more depths in a single day, decreasing the number of replicates and the number of dilutions would permit us to do a few depths each week and these results could be compared to those of the week after, or to measurements in other seasons, so that both spatial and temporal variability are analysed.

The dilution curves based on chlorophyll *a*, sometimes indicated saturated feeding for the microzooplankton. To account for this we could have used the “3-point” method of Gallegos (1989) to estimate growth. With this method only the most dilute treatments are used to extrapolate for phytoplankton growth at infinite dilution. Using this method can sometimes give more accurate results. However, due to time restrictions further re-analysis of the data will be conducted post thesis completion and prior to submitting the research for publication in a journal.

More information about the grazing in function of the cell volumes may be obtained by a fractionating filtration, where the water is filtered through two filters of different sizes and the impact of grazing on different class size may so be measured.

An improved, but more laborious, technique to increase the ecological relevance of such experiments, would be to include a measure of the impact of grazing by macrozooplankton too. In fact, the dilution method can overestimate microzooplankton grazing rates as a result of the exclusion of zooplankton > 200 µm from the experiment bottles. This overestimation can be considerable when, for example, copepod predation is high on ciliates (Atkinson 1996). Larger metazoans are not rendered irrelevant by a more uniform dominance of microherbivory in the oceans (Calbet 2001), however, their different roles as exporters of euphotic zone production, as trophic connections to fish stocks, and structuring agents of the marine plankton, need to be kept in perspective.

In fact, macrozooplankton are well known to be vertically migrating in order to escape visual predators (such as fish) and their behaviours may drive changes in microzooplankton dynamics and so on the DVM of phytoplankton. This trophic cascade should be analysed at different levels on the same time so that the contribution of each level is correctly estimated, in presence of preys and predators.

Part 2 : Does the presence of zooplankton stimulate the vertical migration of *Gymnodinium catenatum*?

The experiment conducted seemed to give a negative answer to this question, even if some variation could be observed. However, no firm conclusion can be drawn as the number of columns was not sufficient to undertake appropriate statistical tests on the data.

For this experiment, several perspectives can be given: the number of columns should first be increased and should reach high numbers so that statistical analyses are possible on the data.

Secondly, we should find a way to ensure that zooplankton is not dying during the experiment. Changing the zooplankton more often could be a solution, however, the laborious work given by the sampling and the isolation of zooplankton unable us to do this, in this study.

Moreover, the experiment could start directly if we knew if the acclimatation period was necessary. We could increase the duration of the experiment with zooplankton in the cages by spending less time to evaluate the impact of the cages on phytoplankton's behaviour.

Finally, this kind of experiment asks an exact knowledge of phytoplankton and zooplankton behaviour. The effects of some materials used or conditions created are not exactly known. For example, putting the zooplankton in the cages could have an effect on their behaviour.

The problem of contamination should be resolved too. In fact, working with such volumes unable us to work under aseptic conditions once the volumes reached are too important. We could imagine that a kind of antibiotics may have been added to the water in the columns so that no bacteria or other contaminator was present.

Chapter 5: References

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